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(54) **MODIFYING SOYBEAN OIL COMPOSITION THROUGH TARGETED KNOCKOUT OF THE FAD2-1A/1B GENES**

(71) Applicant: **CELLECTIS**, Paris (FR)

(72) Inventors: **Luc Mathis**, Le Kremlin Bicetre (FR);
Daniel F. Voytas, Falcon Heights, MN (US);
Feng Zhang, Plymouth, MN (US);
William Haun, St. Paul, MN (US)

(73) Assignee: **CELLECTIS**, Paris (FR)

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(58) **Field of Classification Search**

None
See application file for complete search history.

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Primary Examiner — Medina A Ibrahim
Assistant Examiner — Wayne Zhong
(74) *Attorney, Agent, or Firm* — Fish & Richardson P.C.

(57) **ABSTRACT**

Materials and methods are provided for making soybean varieties that have altered oil composition as a result of mutations in the FAD2-1A and FAD2-1B genes.

13 Claims, 6 Drawing Sheets

Specification includes a Sequence Listing.

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Figure 1A

FAD2_1A_T1
TTCAATTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCAACCCCTTTTCcctcaTTGCATGGCCCAATCTATTGGGTTCTCCAAAGGTTGCCCTTCTCACTGGTGTGGGTGATTGCTCACGAGTGTGGTCACCATGCCCTTCAGCAAGTACCAATGGGTTGATGATGTGTGGGTTGACCCCTTCACTCAACACTTTTAGTCCCTTATTCTCATGGAAAAATAAGCCATCGccgcCATC
ACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1A_T2
TTCAATTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCAACCCCTTTTCcctcaTTGCATGGCCCAATCTATTGGGTTCTCCAAAGGTTGCCCTTCTCACTGGTGTGGGTGATTGCTCACGAGTGTGGTCACCATGCCCTTCAGCAAGTACCAATGGGTTGATGATGTGTGGGTTGACCCCTTCACTCAACACTTTTAGTCCCTTATTCTCATGGAAAAATAAGCCATCGccgcCATC
ACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1A_T3
TTCAATTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCAACCCCTTTTCcctcaTTGCATGGCCCAATCTATTGGGTTCTCCAAAGGTTGCCCTTCTCACTGGTGTGGGTGATTGCTCACGAGTGTGGTCACCATGCCCTTCAGCAAGTACCAATGGGTTGATGATGTGTGGGTTGACCCCTTCACTCAACACTTTTAGTCCCTTATTCTCATGGAAAAATAAGCCATCGccgcCATC
ACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1A_T4
TTCAATTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCAACCCCTTTTCcctcaTTGCATGGCCCAATCTATTGGGTTCTCCAAAGGTTGCCCTTCTCACTGGTGTGGGTGATTGCTCACGAGTGTGGTCACCATGCCCTTCAGCAAGTACCAATGGGTTGATGATGTGTGGGTTGACCCCTTCACTCAACACTTTTAGTCCCTTATTCTCATGGAAAAATAAGCCATCGccgcCATC
ACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

Figure 1B

FAD2_1B_T1
TTCATTTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCACCCTTTTCCCTCATTGTCATGGCCAAATCTATTGGGTICT
CCAAGGTTGCATTTACTGGCGTGTGGTGATTTGCTCAGGAGTGTTGGTCACCATGCCCTTCAGCAAGTACCCCATGGGTT
GATGATGTTATGGGTTTGACCGTTCACATCAGCACTTTATGTCCTTATTCTCATGGAAAAATAAGCCATCGccgcCACCACCT
CCAACACGGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1B_T2
TTCATTTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCACCCTTTTCCCTCATTGTCATGGCCAAATCTATTGGGTICT
CCAAGGTTGCATTTACTGGCGTGTGGTGATTTGCTCAGGAGTGTTGGTCACCATGCCCTTCAGCAAGTACCCCATGGGTT
GATGATGTTATGGGTTTGACCGTTCACATCAGCACTTTATGTCCTTATTCTCATGGAAAAATAAGCCATCGccgcCACCACCT
CCAACACGGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1B_T3
TTCATTTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCACCCTTTTCCCTCATTGTCATGGCCAAATCTATTGGGTICT
CCAAGGTTGCATTTACTGGCGTGTGGTGATTTGCTCAGGAGTGTTGGTCACCATGCCCTTCAGCAAGTACCCCATGGGTT
GATGATGTTATGGGTTTGACCGTTCACATCAGCACTTTATGTCCTTATTCTCATGGAAAAATAAGCCATCGccgcCACCACCT
CCAACACGGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

FAD2_1B_T4
TTCATTTTCTACATTGCCACCACCTACTTCCACCTCCCTCCCTCACCCTTTTCCCTCATTGTCATGGCCAAATCTATTGGGTICT
CCAAGGTTGCATTTACTGGCGTGTGGTGATTTGCTCAGGAGTGTTGGTCACCATGCCCTTCAGCAAGTACCCCATGGGTT
GATGATGTTATGGGTTTGACCGTTCACATCAGCACTTTATGTCCTTATTCTCATGGAAAAATAAGCCATCGccgcCACCACCT
CCAACACGGGTTCCCTTGACCGTGATGAAGTGTGTGTCCTCCAAAAC

Figure 2

Target		Treatment	
FAD2-1A	FAD2_T01	a b c WT	a b c d e f WT
	FAD2_T04	a b c WT	a b c d e f WT
FAD2-1B	FAD2_T01	A B	A B
	FAD2_T04	C D	C D

Figure 3

	Deletions/ Inserts	SEQ ID NO:
FAD2-1A (FAD2_T1_C40)		
<u>GCCACCCTACTTCCACCCTCCCTTCCCTCAACCCCTTTCCCTCATTGCATGGCCAAATCTATTGGGTTCTCCAAGGTT</u>	0	3
<u>GCCACCCTACTTCCACCCTCCCTCCCTCAA</u> -----GATT-----GCCAAATCTATTGGGTTCTCCAAGGTT	-16/+4	4
<u>GC</u> -----ATGCCAAATCTATTGGGTTCTCCAAGGTT	-45	5
FAD2-1B (FAD2_T1_C40)		
<u>GCCACCCTACTTCCACCCTCCCTCCCTCACCCTTTCCCTCATTGCATGGCCAAATCTATTGGGTTCTCCAAGGTT</u>	0	3
<u>GCCACCCTACTTCCACCCTCCCTCCCTCA</u> -----TTGCATGGCCAAATCTATTGGGTTCTCCAAGGTT	-14	6
FAD2-1A (FAD2_T4_C11)		
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTC</u>	0	7
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTCAACAC</u> -----TTGACCGTGATGAAGTGTGTGTC	-8	8
<u>ATTTCTCATGGAAAAATAAGCCATCGCCG</u> -----GATGAAGTGTGTGTC	-30	9
FAD2-1B (FAD2_T4_C11)		
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTGTC</u>	0	7
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTCAACAC</u> -----CCTTGACCGTGATGAAGTGTGTGTC	-6	10
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTCAACAC</u> -----TGAAGTGTGTGTC	-92	11
<u>ATTTCTCATGGAAAAATAAGCCATCGCCGCCATCCTTGACCGTGATGAAGTGTGTGTC</u>	-22	12

Figure 4

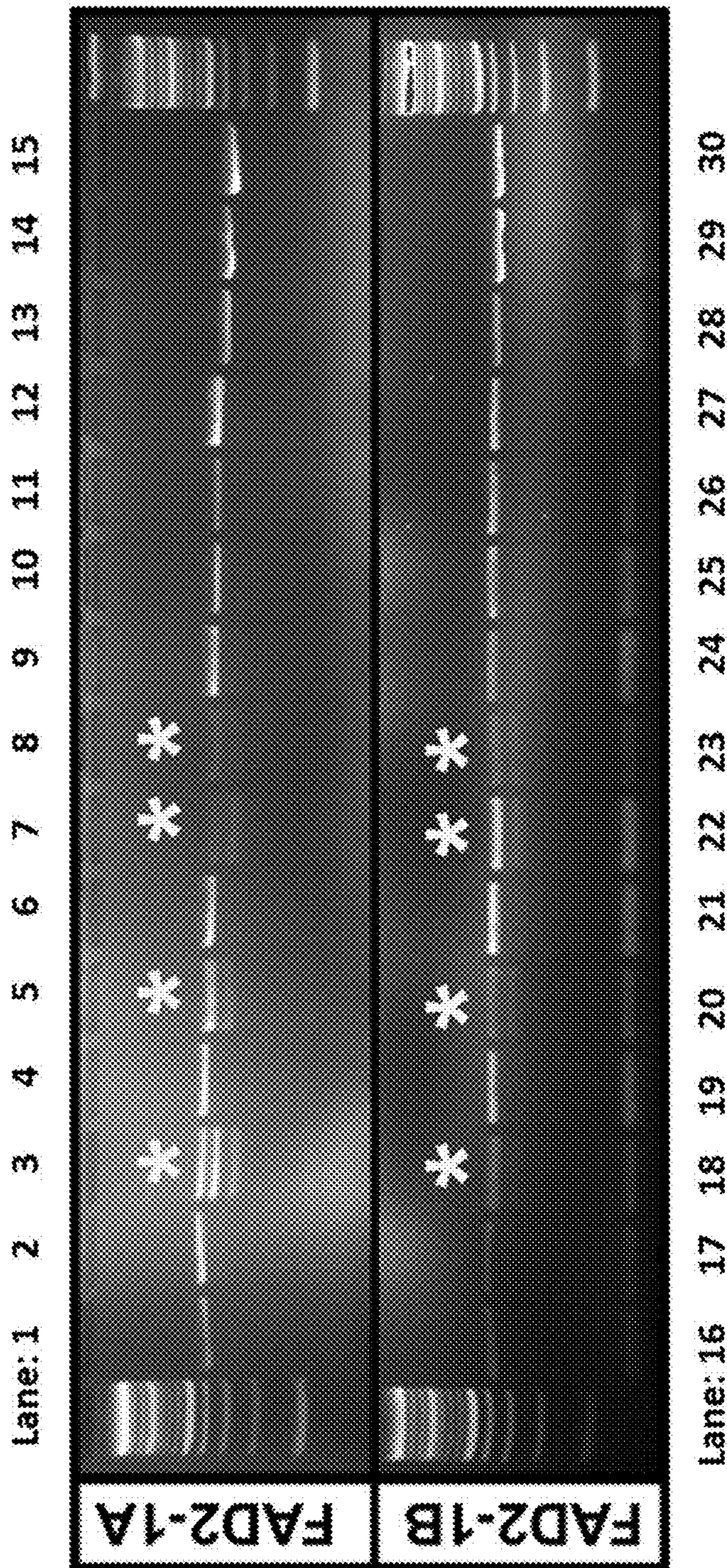


Figure 5

GM026-18		SEQ ID NO:
Sequence (FAD2-1A)		
ATTTCTCATGGAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTCCCTTGACCCGTGATGAAGTGTGTGCCA		13
-----CTTGACCCGTGATGAAGTGTGTGCCA		14
Sequence (FAD2-1B)		
ATTTCTCATGGAAAATAAGCCATCGCCGCCACCACTCCAACACGGGTTCCCTTGACCCGTGATGAAGTGTGTGCCA		15
ATTTCTCATGGAAAATAAGCCATCGCC-----CTTGACCCGTGATGAAGTGTGTGTGCCA		16
GM026-23		
Sequence (FAD2-1A)		
ATTTCTCATGGAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTCCCTTGACCCGTGATGAAGTGTGTGCCA		13
ATTTCTCATGGAAAATAAGCCATCGCC-----T-----CAGGTTCCCTTGACCCGTGATGAAGTGTGTGTGCCA		17
GM027-06		
Sequence (FAD2-1B)		
ATTTCTCATGGAAAATAAGCCATCGCCGCCACCACTCCAACACGGGTTCCCTTGACCCGTGATGAAGTGTGTGCCA		15
ATTTCTCATGGAAAATAAGCCATCGCC-----CTTGACCCGTGATGAAGTGTGTGTGCCA		16

**MODIFYING SOYBEAN OIL COMPOSITION
THROUGH TARGETED KNOCKOUT OF
THE FAD2-1A/1B GENES**

CROSS-REFERENCE TO RELATED
APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application Ser. No. 61/790,655, filed on Mar. 15, 2013.

TECHNICAL FIELD

This document relates to materials and methods for making soybean plants that can be used to produce oil having a modified composition as compared to wild type plants. This document also relates to soybean varieties that lack FAD2-1A/1B activity.

BACKGROUND

Soybean (*Glycine max*) is an important legume crop worldwide due to its ability to fix atmospheric nitrogen. Soybeans also serve as a major source of animal feed protein, and its oil has uses ranging from cooking/frying to industrial uses and biodiesel. Typically, a hydrogenation process is used to increase heat stability and improve shelf life and taste of soybean oil. However, hydrogenation increases the cost of production and also results in the formation of trans fats, which have been linked to cardiovascular disease in humans.

SUMMARY

Provided herein are materials and methods for modifying soybean oil composition by reducing or eliminating expression of the delta-twelve fatty acid desaturase 2 (FAD2) 1A and 1B genes without the use of transgenesis. Soybean varieties having such modified oil composition also are provided.

The methods described herein utilize sequence-specific, rare-cutting endonucleases to introduce mutations in the FAD2-1A and/or FAD2-1B coding sequences, thereby knocking out gene function. These methods mediate FAD2-1A/1B silencing without insertion of a transgene, which would not be acceptable in Europe and other regions of the world. The methods described herein also can be more cost-effective than transgenic approaches.

In one aspect, this document features a soybean plant, plant part, or plant cell having a mutation in one or more FAD2-1A alleles, a mutation in one or more FAD2-1B alleles, or a mutation in one or more FAD2-1A alleles and a mutation in one or more FAD2-1B alleles, wherein the plant, plant part, or plant cell produces oil that has increased oleic acid content and decreased linoleic acid content as compared to oil produced from a corresponding wild type soybean plant, plant part, or plant cell. Each mutation can be at a sequence in SEQ ID NO:1 or SEQ ID NO:2. Each mutation can be induced by a rare-cutting endonuclease (e.g., a transcription activator-like (TAL) effector endonuclease). The TAL effector endonuclease can bind to a sequence as set forth in any of SEQ ID NOS:18-33. Each mutation can be a deletion of more than one nucleotide. Each FAD2-1A and FAD2-1B allele can have a deletion of an endogenous nucleic acid sequence, without including any exogenous nucleic acid. The plant part can be a seed.

In another aspect, this document features a soybean plant, plant part, or plant cell having a mutation in one or more FAD2-1A alleles, a mutation in one or more FAD2-1B alleles, or a mutation in one or more FAD2-1A alleles and a mutation in one or more FAD2-1B alleles, wherein the plant, plant part, or plant cell produces oil that has an oleic acid content of greater than 30% (e.g., greater than 40%, greater than 50%, or greater than 55%).

In another aspect, this document features a soybean plant, plant part, or plant cell having a mutation in one or more FAD2-1A alleles, a mutation in one or more FAD2-1B alleles, or a mutation in one or more FAD2-1A alleles and a mutation in one or more FAD2-1B alleles, wherein the plant, plant part, or plant cell produces oil that has a linoleic acid content of less than 10% (e.g., less than 5%, less than 3%, or less than 1%).

In another aspect, this document features a method for producing soybean oil having increased oleic acid content and reduced linoleic acid content. The method can include (a) providing a soybean plant or plant part having a mutation in one or more FAD2-1A alleles, a mutation in one or more FAD2-1B alleles, or a mutation in one or more FAD2-1A alleles and a mutation in one or more FAD2-1B alleles; and (b) producing oil from the plant or plant part. Each mutation can be induced by a rare-cutting endonuclease (e.g., a TAL effector endonuclease). The TAL effector endonuclease can bind to a sequence as set forth in any of SEQ ID NOS:18-33.

In still another aspect, this document features a method for making a soybean plant having a mutation in each FAD2-1A allele and a mutation in each FAD2-1B allele. The method can include (a) contacting a population of soybean plant cells having functional FAD2-1A and FAD2-1B alleles with one or more rare-cutting endonucleases targeted to endogenous FAD2-1A sequences, and one or more rare-cutting endonucleases targeted to endogenous FAD2-1B sequences, (b) selecting, from the population, a cell in which each FAD2-1A allele and each FAD2-1B allele has been inactivated, and (c) regenerating the selected plant cell into a soybean plant. The soybean plant cells can contain cotyledon cells. The method can include transforming cotyledon cells with one or more vectors encoding the one or more rare-cutting endonucleases. The one or more rare-cutting endonucleases can be TAL effector endonucleases. Each of the TAL effector endonucleases can be targeted to a sequence as set forth in any of SEQ ID NOS:18-33. The method can include introducing into the plant cells one or more TAL effector endonuclease proteins. The method can further include culturing the plant cells to generate plant lines. The method can further include isolating genomic DNA containing at least a portion of the FAD2-1A locus or at least a portion of the FAD2-1B locus from the plant cells.

This document also features a method for generating a soybean plant having a mutation in each FAD2-1A allele and a mutation in each FAD2-1B allele. The method can include (a) crossing a first soybean plant having a mutation in at least one FAD2-1A allele and a mutation in at least one FAD2-1B allele with a second soybean plant having a mutation in at least one FAD2-1A allele and a mutation in at least one FAD2-1B allele, to obtain progeny; and (b) selecting from the progeny a soybean plant that has a mutation in each FAD2-1A and FAD2-1B allele. Each mutation can be induced by a rare-cutting endonuclease (e.g., a TAL effector endonuclease). The TAL effector endonuclease can bind to a sequence as set forth in any of SEQ ID NOS:18-33. Each mutation can be a deletion of more than one nucleotide.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly

understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 shows DNA sequences for various TAL effector endonuclease target sites in FAD2-1A and FAD2-1B. DNA sequence from the FAD2-1A gene (SEQ ID NO:1) is shown in FIG. 1A, and DNA sequence from the FAD2-1B gene (SEQ ID NO:2) is shown in FIG. 1B. The underlined sequences indicate target sites for TAL effector endonucleases. Lower case letters denote restriction endonuclease sites used to screen for TAL effector endonuclease-induced mutations.

FIG. 2 is a picture of a gel showing products from a PCR enrichment assay used to screen soybean hairy roots for TAL effector endonuclease-induced mutations in FAD2-1A and FAD2-1B.

FIG. 3 shows exemplary DNA sequences of TAL effector endonuclease-induced mutations in the FAD2-1A and FAD2-1B genes in soybean hairy roots. The top line of each panel shows the DNA sequence of the recognition site for the TAL effector endonucleases (underlined) in FAD2-1A (top two panels) and FAD2-1B (bottom two panels). The other sequences show representative mutations that were induced by imprecise non-homologous end joining (NHEJ), with the sizes of deletions given on the right.

FIG. 4 is a picture of a gel showing the result of a T7E1 assay performed on regenerated soybean plants to identify TAL effector endonuclease-induced mutations in FAD2-1A and FAD2-1B. Table 4A herein provides information about which regenerated plant and which particular FAD2-1 gene were analyzed in each lane of the gel. The first and last lanes are molecular length markers and are not numbered. DNA samples listed as "uncut" in Table 4A were not treated with T7E1.

FIG. 5 shows DNA sequences of TAL effector endonuclease-induced mutations in FAD2-1A and FAD2-1B. These mutations were genetically transmissible.

DETAILED DESCRIPTION

Commodity soybean oil is made up of five fatty acids: palmitic acid (10%), stearic acid (4%), oleic acid (18%), linoleic acid (55%) and linolenic acid (13%). Plant oils with high oleic acid content may require less processing to improve stability and/or taste. Such oils also may be healthier, as well as better suited for the production of biodiesel. Soybean oil having an oleic acid content of greater than 55% and a linoleic acid content of less than 10% can be particularly useful. Traditional breeding and mutagenesis strategies have been used to generate soybean varieties

containing elevated levels of oleic acid, but these varieties have reduced yield and thus have not been acceptable to farmers.

The enzymes responsible for the biosynthetic progression from palmitic acid to linolenic acid have been identified. For example, the FAD2 genes are responsible for converting oleic acid precursors to linoleic acid precursors during oil accumulation in developing soybean seeds. Due to the ancient polyploidization of soybean, two copies of FAD2 (FAD2-1A and FAD2-1B) exist in the soybean genome. These genes have about 95% sequence identity at the DNA level, and the encoded proteins have about 99% sequence identity at the amino acid level. Plants homozygous for naturally occurring FAD2-1B mutant alleles can have a modest increase (20.5% to 29.4%) in oleic acid composition, as described elsewhere (Pham et al., *BMC Plant Biol.* 10:195, 2010). Mutations in FAD2-1A have been developed through X-ray mutagenesis and TILLing, to produce seeds containing up to 50% oleic acid (Sandhu et al., *JAOC* 84:229-235, 2007), and mutating both the FAD2-1A and FAD2-1B alleles resulted in oil with an oleic acid content of 82.2% (Pham et al., *supra*).

This document provides soybean plants that have reduced (e.g., lack) FAD2-1A and/or FAD2-1B activity, as well methods for generating such plants, and oil derived from such plants. The methods described herein can be used to generate soybean varieties having oil with an increased oleic acid component of at least 30% (e.g., at least 35%, at least 40%, at least 45%, at least 50%, or at least 55%, and a reduction in linoleic acid component to 10% or less (e.g., 8% or less, 5% or less, 4% or less, 3% or less, 2% or less, 1% or less, or 0.5% or less). In some embodiments, this modification of soybean oil composition can be achieved by completely knocking out the expression of the FAD2-1A and/or FAD2-1B genes. According to some of the methods provided herein, both alleles of FAD2-1A and/or FAD2-1B genes are inactivated using non-transgenic techniques. Removing the all RNA transcripts of FAD2-1A/1B can severely reduce the conversion of oleic acid precursors to linoleic acid precursors in soybean seeds.

To accomplish the complete elimination of FAD2-1A/1B expression, for example, an engineered, rare-cutting nuclease was designed to recognize a conserved region of both FAD2-1 genes and create a double-strand break. Improper repair due to Non-Homologous End Joining (NHEJ) at the DNA break site generates missense and/or nonsense mutations in the FAD2-1A/1B coding regions, rendering the FAD2-1A/1B RNA transcripts unstable and targeted for degradation prior to translation.

In soybean, there are at least two members (1A and 1B) in the FAD2 gene family. Representative examples of naturally occurring soybean FAD2-1A and FAD2-1B nucleotide sequences are shown in Table 5. The soybean plants, cells, plant parts, seeds, and progeny thereof provided herein can have a mutation in each of the endogenous FAD2-1A and FAD2-1B alleles, such that expression of each gene is reduced or completely inhibited. Alternatively, the soybean plants, cells, plant parts, seeds, and progeny thereof provided herein may have a mutation in at least one FAD2-1A allele and/or in at least one FAD2-1B allele, such that expression of each gene is reduced or completely inhibited. The soybean plants, cells, parts, seeds, and progeny can have increased levels of oleic acid and reduced levels of linoleic acid as compared to wild type soybean plants, cells, parts, seeds, and progeny.

The plants, plant cells, plant parts, seeds, and plant progeny provided herein can be generated using a TAL

effector endonuclease system to make targeted knockouts in the FAD2-1A and/or FAD2-1B genes. Thus, this disclosure provides materials and methods for using TAL effector endonucleases to generate plants and related products (e.g., seeds and plant parts) that are particularly suitable for production of soybean oil with increased oleic acid content and decreased linoleic acid content. Other rare cutting, sequence-specific nucleases can be used to generate the desired plant material, including engineered homing endonucleases or zinc finger nucleases.

“Plants” and “plant parts” refers to cells, tissues, organs, seeds, and severed parts (e.g., roots, leaves, and flowers) that retain the distinguishing characteristics of the parent plant. “Seed” refers to any plant structure that is formed by continued differentiation of the ovule of the plant, following its normal maturation point at flower opening, irrespective of whether it is formed in the presence or absence of fertilization and irrespective of whether or not the seed structure is fertile or infertile.

The term “allele(s)” means any of one or more alternative forms of a gene at a particular locus. In a diploid (or amphidiploid) cell of an organism, alleles of a given gene are located at a specific location or locus on a chromosome. One allele is present on each chromosome of the pair of homologous chromosomes. “Heterozygous” alleles are two different alleles residing at a specific locus, positioned individually on corresponding pairs of homologous chromosomes. “Homozygous” alleles are two identical alleles residing at a specific locus, positioned individually on corresponding pairs of homologous chromosomes in the cell.

“Wild type” as used herein refers to a typical form of a plant or a gene as it most commonly occurs in nature. A “wild type FAD2-1A allele” is a naturally occurring FAD2-1A allele (e.g., as found within naturally occurring soybean plants) that encodes a functional FAD2-1A protein, while a “mutant FAD2-1A allele” is a FAD2-1A allele that does not encode a functional FAD2-1A protein. Such a “mutant FAD2-1A allele” can include one or more mutations in its nucleic acid sequence, where the mutation(s) result in no detectable amount of functional FAD2-1A protein in the plant or plant cell in vivo.

The term “rare-cutting endonucleases” herein refer to natural or engineered proteins having endonuclease activity directed to nucleic acid sequences having a recognition sequence (target sequence) about 12-40 bp in length (e.g., 14-40 bp in length). Typical rare-cutting endonucleases cause cleavage inside their recognition site, leaving 4 nt staggered cut with 3'OH or 5'OH overhangs. These rare-cutting endonucleases may be meganucleases, such as wild type or variant proteins of homing endonucleases, more particularly belonging to the dodecapeptide family (LAGLI-DADG (SEQ ID NO:37); see, WO 2004/067736) or may result from fusion proteins that associate a DNA binding domain and a catalytic domain with cleavage activity. TAL-effector endonucleases and zinc-finger-nucleases (ZFN) are examples of fusions of DNA binding domains with the catalytic domain of the endonuclease FokI. Customized TAL effector endonucleases are commercially available under the trade name TALEN™ (Collectis, Paris, France). For a review of rare-cutting endonucleases, see Baker, *Nature Methods* 9:23-26, 2012.

“Mutagenesis” as used herein refers to processes in which mutations are introduced into a selected DNA sequence. Mutations induced by endonucleases generally are obtained by a double strand break, which results in in-deletions (“indels”) that can be detected by deep-sequencing analysis. Such mutations typically are deletions of several base pairs,

and have the effect of inactivating the mutated allele. In the methods described herein, for example, mutagenesis occurs via double stranded DNA breaks made by TAL effector endonucleases targeted to selected DNA sequences in a plant cell. Such mutagenesis results in “TAL effector endonuclease-induced mutations” (e.g., TAL effector endonuclease-induced knockouts) and reduced expression of the targeted gene. Following mutagenesis, plants can be regenerated from the treated cells using known techniques (e.g., planting seeds in accordance with conventional growing procedures, followed by self-pollination).

In some cases, a nucleic acid can have a nucleotide sequence with at least about 75 percent sequence identity to a representative FAD2-1A or FAD2-1B nucleotide sequence. For example, a nucleotide sequence can have at least 75, at least 80, at least 85, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, or at least 99 percent sequence identity to a representative, naturally occurring FAD2-1A or FAD2-1B nucleotide sequence as set forth in Table 3.

The percent sequence identity between a particular nucleic acid or amino acid sequence and a sequence referenced by a particular sequence identification number is determined as follows. First, a nucleic acid or amino acid sequence is compared to the sequence set forth in a particular sequence identification number using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained online at fr.com/blast or at ncbi.nlm.nih.gov. Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence (e.g., SEQ ID NO:1), or by

an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 250 matches when aligned with the sequence set forth in SEQ ID NO:1 is 86.5 percent identical to the sequence set forth in SEQ ID NO:1 (i.e., $250 \div 289 \times 100 = 86.5$). It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 is rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 is rounded up to 75.2. It also is noted that the length value will always be an integer.

Methods for selecting endogenous target sequences and generating TAL effector endonucleases targeted to such sequences can be performed as described elsewhere. See, for example, PCT Publication No. WO 2011/072246 (which is incorporated herein by reference in its entirety). TAL effectors are found in plant pathogenic bacteria in the genus *Xanthomonas*. These proteins play important roles in disease, or trigger defense, by binding host DNA and activating effector-specific host genes (see, e.g., Gu et al., *Nature* 435:1122-1125, 2005; Yang et al., *Proc. Natl. Acad. Sci. USA* 103:10503-10508, 2006; Kay et al. *Science* 318:648-651, 2007; Sugio et al., *Proc. Natl. Acad. Sci. USA* 104:10720-10725, 2007; and Römer et al. *Science* 318:645-648, 2007). Specificity depends on an effector-variable number of imperfect, typically 34 amino acid repeats (Schornack et al., *J. Plant Physiol.* 163:256-272, 2006; and WO 2011/072246). Polymorphisms are present primarily at repeat positions 12 and 13, which are referred to herein as the repeat variable-diresidue (RVD).

The RVDs of TAL effectors correspond to the nucleotides in their target sites in a direct, linear fashion, one RVD to one nucleotide, with some degeneracy and no apparent context dependence. This mechanism for protein-DNA recognition enables target site prediction for new target specific TAL effectors, as well as target site selection and engineering of new TAL effectors with binding specificity for the selected sites.

TAL effector DNA binding domains can be fused to other sequences, such as endonuclease sequences, resulting in chimeric endonucleases targeted to specific, selected DNA sequences, and leading to subsequent cutting of the DNA at or near the targeted sequences. Such cuts (i.e., double-stranded breaks) in DNA can induce mutations into the wild type DNA sequence via NHEJ or homologous recombination, for example. In some cases, TAL effector endonucleases can be used to facilitate site directed mutagenesis in complex genomes, knocking out or otherwise altering gene function with great precision and high efficiency. As described in the Examples below, TAL effector endonucleases targeted to the soybean FAD2-1A and FAD2-1B alleles can be used to mutagenize the endogenous genes, resulting in plants with reduced expression (e.g., without detectable expression) of these genes. The fact that some endonucleases (e.g., FokI) function as dimers can be used to enhance the target specificity of the TAL effector endonuclease. For example, in some cases a pair of TAL effector endonuclease monomers targeted to different DNA sequences (e.g., the underlined target sequences shown in FIGS. 1A and 1B) can be used. When the two TAL effector endonuclease recognition sites are in close proximity, as depicted in FIGS. 1A and 1B, the inactive monomers can come together to create a functional enzyme that cleaves the DNA. By requiring DNA binding to activate the nuclease, a highly site-specific restriction enzyme can be created.

The term “expression” as used herein refers to the transcription of a particular nucleic acid sequence to produce sense or antisense mRNA, and/or the translation of a sense mRNA molecule to produce a polypeptide (e.g., a therapeutic protein), with or without subsequent post-translational events.

“Reducing the expression” of a gene or polypeptide in a plant or a plant cell includes inhibiting, interrupting, knocking-out, or knocking-down the gene or polypeptide, such that transcription of the gene and/or translation of the encoded polypeptide are reduced as compared to a corresponding wild type plant or plant cell. Expression levels can be assessed using methods such as, for example, reverse transcription-polymerase chain reaction (RT-PCR), Northern blotting, dot-blot hybridization, in situ hybridization, nuclear run-on and/or nuclear run-off, RNase protection, or immunological and enzymatic methods such as ELISA, radioimmunoassay, and western blotting.

Methods for using TAL effector endonucleases to generate plants, plant cells, or plant parts having mutations in endogenous genes include, for example, those described in the Examples herein. For example, nucleic acids encoding TAL effector endonucleases targeted to selected FAD2-1A or FAD2-1B sequences (e.g., the FAD2-1A sequences shown in FIG. 1A or the FAD2-1B sequences shown in FIG. 1B) can be transformed into plant cells (e.g., cells in cotyledons), where they can be expressed. The cells subsequently can be analyzed to determine whether mutations have been introduced at the target site(s), through nucleic acid-based assays or protein-based assays to detect expression levels as described above, for example, or using nucleic acid-based assays (e.g., PCR and DNA sequencing, or PCR followed by a T7E1 assay; Mussolino et al., *Nucleic Acids Res* 39:9283-9293, 2011) to detect mutations at the genomic loci.

The mutagenized population, or a subsequent generation of that population, can be screened for a desired trait(s) (e.g., plants that have altered oil composition) that results from the mutations. Alternatively, the mutagenized population, or a subsequent generation of that population, can be screened for a mutation in a FAD2-1A or FAD2-1B gene. For example, the progeny M_2 generation of M_1 plants may be evaluated for the presence of a mutation in a FAD2-1A or FAD2-1B gene. A “population” is any group of individuals that share a common gene pool. As used herein, “ M_0 ” refers to plant cells (and plants grown therefrom) exposed to a TAL effector nuclease, while “ M_1 ” refers to seeds produced by self-pollinated M_0 plants, and plants grown from such seeds. “ M_2 ” is the progeny (seeds and plants) of self-pollinated M_1 plants, “ M_3 ” is the progeny of self-pollinated M_2 plants, and “ M_4 ” is the progeny of self-pollinated M_3 plants. “ M_5 ” is the progeny of self-pollinated M_4 plants. “ M_6 ”, “ M_7 ”, etc. are each the progeny of self-pollinated plants of the previous generation. The term “selfed” as used herein means self-pollinated.

One or more M_1 soybean plants can be obtained from individual, mutagenized M_0 plant cells (and plants grown therefrom), and at least one of the plants can be identified as containing a mutation in a FAD2-1A or FAD2-1B gene. An M_1 soybean plant may be heterozygous for a mutant allele at a FAD2-1A and/or a FAD2-1B locus and, due to the presence of the wild-type allele, exhibit delta-twelve fatty acid desaturase activity. Alternatively, an M_1 soybean plant may have a mutant allele at a FAD2-1A or FAD2-1B locus and exhibit the phenotype of lacking delta-twelve fatty acid desaturase activity. Such plants may be heterozygous and lack delta-twelve fatty acid desaturase activity due to phenomena such a dominant negative suppression, despite the

presence of the wild-type allele, or may be homozygous due to independently induced mutations in both alleles at the FAD2-1A or FAD2-1B locus.

A soybean plant carrying mutant FAD2-1A and FAD2-1B alleles can be used in a plant breeding program to create novel and useful lines and varieties. Thus, in some embodiments, an M_1 , M_2 , M_3 , or later generation soybean plant containing at least one mutation in a FAD2-1A and at least one mutation in a FAD2-1B gene is crossed with a second soybean plant, and progeny of the cross are identified in which the FAD2-1A and FAD2-1B gene mutations are present. It will be appreciated that the second soybean plant can contain the same FAD2-1A and FAD2-1B mutations as the plant to which it is crossed, different FAD2-1A and FAD2-1B mutations, or be wild-type at the FAD2-1A and/or FAD2-1B loci.

Breeding can be carried out via known procedures. DNA fingerprinting, SNP or similar technologies may be used in a marker-assisted selection (MAS) breeding program to transfer or breed mutant FAD2-1A and FAD2-1B alleles into other soybean plants. For example, a breeder can create segregating populations from hybridizations of a genotype containing a mutant allele with an agronomically desirable genotype. Plants in the F_2 or backcross generations can be screened using markers developed from FAD2-1A and FAD2-1B sequences or fragments thereof. Plants identified as possessing the mutant allele can be backcrossed or self-pollinated to create a second population to be screened. Depending on the expected inheritance pattern or the MAS technology used, it may be necessary to self-pollinate the selected plants before each cycle of backcrossing to aid identification of the desired individual plants. Backcrossing or other breeding procedure can be repeated until the desired phenotype of the recurrent parent is recovered.

Successful crosses yield F_1 plants that are fertile and that can be backcrossed with one of the parents if desired. In some embodiments, a plant population in the F_2 generation is screened for FAD2-1A and FAD2-1B gene expression, e.g., a plant is identified that fails to express FAD2-1A and FAD2-1B due to the absence of a FAD2-1A and FAD2-1B genes according to standard methods, for example, using a PCR method with primers based upon the nucleotide sequence information for FAD2-1A and FAD2-1B described herein. Selected plants are then crossed with one of the parents and the first backcross (BC_1) generation plants are self-pollinated to produce a BC_1F_2 population that is again screened for variant FAD2-1A and FAD2-1B gene expression (e.g., null versions of the FAD2-1A and FAD2-1B genes). The process of backcrossing, self-pollination, and screening is repeated, for example, at least four times until the final screening produces a plant that is fertile and reasonably similar to the recurrent parent. This plant, if desired, can be self-pollinated, and the progeny subsequently can be screened again to confirm that the plant lacks FAD2-1A and FAD2-1B gene expression. Cytogenetic analyses of the selected plants optionally can be performed to confirm the chromosome complement and chromosome pairing relationships. Breeder's seed of the selected plant can be produced using standard methods including, for example, field testing, confirmation of the null condition for FAD2-1A and FAD2-1B, and/or analyses of oil to determine the level of oleic acid and linoleic acid.

In situations where the original F_1 hybrid resulting from the cross between a first, mutant soybean parent and a second, wild-type soybean parent, is hybridized or backcrossed to the mutant soybean parent, the progeny of the

backcross can be self-pollinated to create a BC_1F_2 generation that is screened for the mutant FAD2-1A and FAD2-1B alleles.

The result of a plant breeding program using the mutant soybean plants described herein can be novel and useful lines and varieties. As used herein, the term "variety" refers to a population of plants that share constant characteristics which separate them from other plants of the same species. A variety is often, although not always, sold commercially. While possessing one or more distinctive traits, a variety can be further characterized by a very small overall variation between individuals within that variety. A "pure line" variety may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques. A variety can be essentially derived from another line or variety. As defined by the International Convention for the Protection of New Varieties of Plants (Dec. 2, 1961, as revised at Geneva on Nov. 10, 1972, on Oct. 23, 1978, and on Mar. 19, 1991), a variety is "essentially derived" from an initial variety if: a) it is predominantly derived from the initial variety, or from a variety that is predominantly derived from the initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety; b) it is clearly distinguishable from the initial variety; and c) except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety. Essentially derived varieties can be obtained, for example, by the selection of a natural or induced mutant, a somaclonal variant, a variant individual from plants of the initial variety, backcrossing, or transformation. A "line" as distinguished from a variety most often denotes a group of plants used non-commercially, for example in plant research. A line typically displays little overall variation between individuals for one or more traits of interest, although there may be some variation between individuals for other traits.

The methods provided herein can be used to produce plant parts (e.g., seeds) or plant products (e.g., oil) having increased oleic acid content and reduced linoleic acid content, as compared corresponding plant parts or products from wild type plants. The fatty acid content of a plant part or a plant product can be evaluated using standard methods, such as those described in Example 5 herein, for example.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1

Engineering Sequence-specific Nucleases to Mutagenize the FAD2-1A and FAD2-1B Genes

To completely inactivate or knock-out the alleles of FAD2-1A and FAD2-1B genes in *G. max*, sequence-specific nucleases were designed that target the protein coding region in the vicinity of the start codon. Eight TAL effector endonuclease pairs were designed to target the FAD2-1 gene family within the first 500 bp of the coding sequence using software that specifically identifies TAL effector endonuclease recognition sites, such as TALE-NT 2.0 (Doyle et al., *Nucleic Acids Res* 40:W117-122, 2012). The TAL effector endonuclease recognition sites for the FAD2-1 genes are

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underlined in FIG. 1 and are listed in Table 1. TAL effector endonucleases were synthesized using methods similar to those described elsewhere (Cermak et al., *Nucleic Acids Res* 39:e82, 2011; Reyon et al., *Nat Biotechnol* 30:460-465, 2012; and Zhang et al., *Nat Biotechnol* 29:149-153, 2011).

Example 2

FAD2-1 TAL Effector Endonuclease Activity in Yeast

To assess the activity of the TAL effector endonucleases targeting the FAD2-1 genes, activity assays were performed in yeast by methods similar to those described elsewhere (Christian et al., *Genetics* 186:757-761, 2010). For these assays, a target plasmid was constructed with the TAL effector endonuclease recognition site cloned in a non-functional β -galactosidase reporter gene (Table 2). The target site was flanked by a direct repeat of β -galactosidase coding sequence such that if the reporter gene was cleaved by the TAL effector endonuclease, recombination would occur between the direct repeats and function would be restored to the β -galactosidase gene. β -galactosidase activity, therefore, served as a measure of TAL effector endonuclease cleavage activity. Results are summarized in Table 3. All of the FAD2-1 TAL effector endonuclease pairs displayed cleavage activity. Cleavage activities were normalized to the benchmark nuclease, I-SceI.

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TABLE 3

FAD2 TAL effector endonuclease activity in yeast				
TAL effector	Activity in yeast*			
endonuclease subunit	FAD2_T1	FAD2_T2	FAD2_T3	FAD2_T4
5 FAD2_T01_Left_C11	0.73	0.87	0.00	0.00
FAD2_T01_Right_C11				
FAD2_T01_Left_C40	0.79	0.73	0.00	0.00
FAD2_T01_Right_C40				
FAD2_T02_Left_C11	0.00	0.97	0.00	0.00
15 FAD2_T01_Right_C11				
FAD2_T02_Left_C40	0.00	0.96	0.00	0.00
FAD2_T01_Right_C40				
FAD2_T03_Left_C11	0.00	0.00	0.65	0.53
20 FAD2_T03_Right_C11				
FAD2_T03_Left_C40	0.00	0.00	0.63	0.51
FAD2_T03_Right_C40				
FAD2_T04_Left_C11	0.00	0.00	0.00	0.54
FAD2_T03_Right_C11				
25 FAD2_T04_Left_C40	0.00	0.00	0.00	0.52
FAD2_T03_Right_C40				

*Normalized to I-SceI (max = 1.0)

TABLE 1

TAL effector endonuclease target sequences				
Gene	Target Sequence Left	SEQ ID NO:	Target Sequence Right	SEQ ID NO
FAD2_T01_C11	GCCACCACCTACTTCCACCTCCT	18	ATTGCATGGCCAATCT	19
FAD2_T01_C40	GCCACCACCTACTTCCACCTCCT	20	ATTGCATGGCCAATCT	21
FAD2_T02_C11	ACATTGCCACCACCTACTTCCACCT	22	ATTGCATGGCCAATCT	23
FAD2_T02_C40	ACATTGCCACCACCTACTTCCACCT	24	ATTGCATGGCCAATCT	25
FAD2_T03_C11	CTCATGGAAAATAAGCCAT	26	ACCGTGATGAAGTGTGTTGTCCC	27
FAD2_T03_C40	CTCATGGAAAATAAGCCAT	28	ACCGTGATGAAGTGTGTTGTCCC	29
FAD2_T04_C11	ATTTCTCATGGAAAATAAGCCAT	30	ACCGTGATGAAGTGTGTTGTCCC	31
FAD2_T04_C40	ATTTCTCATGGAAAATAAGCCAT	32	ACCGTGATGAAGTGTGTTGTCCC	33

TABLE 2

FAD2 TAL effector endonuclease Target Sequences for Yeast Assay		
TAL effector endonuclease target	TAL effector endonuclease target sequence	SEQ ID NO:
FAD2 Target 1	GCCACCACCTACTTCCACCTCCTTCTCAACCCTTTTCCCTCATTGCATGGCCAATCT	34
FAD2 Target 2	ACATTGCCACCACCTACTTCCACCTCCTTCTCAACCCTTTTCCCTCATTGCATGGCCAATCT	35
FAD2 Target 3	CTCATGGAAAATAAGCCATCGCCGCATCACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTTGTCCC	36
FAD2 Target 4	ATTTCTCATGGAAAATAAGCCATCGccgcCATCACTCCAACACAGGTTCCCTTGACCGTGATGAAGTGTGTTGTCCC	7

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Example 3

TAL Effector Endonuclease Expression and Activity in Soybean Hairy Roots

Based on the activity of the various FAD2 TAL effector endonucleases in yeast, four TAL effector endonucleases in two different scaffolds were chosen for expression in soybean cells (FAD2_T01, FAD2_T02, FAD2_T03 and FAD2_T04). The activities of these TAL effector endonucleases were assessed at their endogenous target sites in soybean using a hairy root assay described elsewhere (Curtin et al. *Plant Physiology* 156(2):466-473, 2011). First, each TAL effector endonuclease was cloned into a T-DNA vector downstream of an estradiol-inducible promoter (Zuo et al., *Plant J.* 24:265-273, 2000), and then transformed into the K599 strain of *Agrobacterium rhizogenes* (Govindarajulu et al., *Mol. Plant Microbe Interact.* 21:1027-1035, 2008). *A. rhizogenes* strains with the various TAL effector endonucleases were then used to infect half-cotyledons of soybean and produce transgenic hairy roots. Three weeks after infection, hairy roots were collected and frozen in liquid nitrogen, and genomic DNA was prepared using standard methods (Murray et al., *Nucl. Acids Res.* 8(19):4321-4325, 1980).

To determine if NHEJ-mediated mutations were created by the TAL effector endonucleases at the target sites in the soybean genome, DNA from nine hairy roots were subjected to a PCR enrichment assay (Zhang et al., *Proc. Natl. Acad. Sci. USA* 107(26):12028-12033, 2010). This assay monitors loss of a restriction enzyme site within the TAL effector endonuclease spacer sequence due to NHEJ-induced mutations. The restriction enzyme sites used in this assay are shown in FIG. 1 (signified by lower case letters). Genomic DNA derived from three hairy root samples expressing FAD2_T01 was digested with MnlI to monitor mutations at the FAD2-1A locus. HphI was used to monitor mutations at FAD2-1B caused by FAD2_T01. Acil was used to monitor mutations at both the FAD2-1A and FAD2-1B loci in digested genomic DNA from six hairy root samples expressing FAD2_T04. Following digestion, an aliquot of each reaction was used as a template for PCR with primers flanking either the FAD2_T01 or FAD2_T04 target sites. To assess activity of FAD2_T01, separate PCR reactions were conducted with primers specific for FAD2-1A and FAD2-1B. PCR products were first analyzed on a 1% agarose gel to verify that amplification had occurred, and then digested with the restriction enzyme located in the spacer sequence of the particular TAL effector endonuclease being analyzed. This second restriction digestion reaction was then analyzed on a 2% agarose gel, along with digested and undigested control DNA from wild type samples. Samples with TAL effector endonuclease-induced NHEJ mutations may lack the restriction enzyme site within the spacer sequence, resulting in an undigested PCR product which appears as a full-length band on the gel. As shown in FIG. 2, undigested PCR products were observed for FAD2-1A, FAD2-1B, or both genes in the same sample. For example, sample "b" in FIG. 2C shows that mutations were present in FAD2-1B, sample "d" in FIG. 2B shows that mutations were present in FAD2-1A, and sample "f" in FIGS. 2B and 2D shows that mutations were present in both FAD2-1A and FAD2-1B.

Undigested PCR products were cloned and sequenced to verify that they contained TAL effector endonuclease-induced mutations in the spacer sequence. The PCR products were cloned using the Qiagen TOPO cloning kit according to manufacturer's instructions. Individual clones derived from a given undigested fragment were sequenced, and the

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DNA sequences aligned with the wild type FAD2-1A or FAD2-1B sequences. As shown in FIG. 3, multiple, independent deletions were recovered in both FAD2-1A and FAD2-1B, ranging in size from 6 to 92 base pairs.

Example 4

Regeneration of Soybean Plants with TAL Effector Endonuclease-induced Mutations in FAD2 Genes

Following verification that FAD2_TAL1 and FAD2_TAL4 created targeted modifications at endogenous target sites, experiments were conducted to create soybean plants with mutations in FAD2-1A and FAD2-1B. To accomplish this, each of the four FAD2-1 TAL effector endonucleases were cloned into a T-DNA vector, and TAL effector endonuclease expression was driven by either the cauliflower mosaic virus 35S promoter or the estradiol-induced XVE promoter system (Zuo et al., supra). The T-DNA vector also contained a bar selectable marker that confers resistance to glufosinate.

Transgenic soybean plants expressing the TAL effector endonucleases were generated using standard *Agrobacterium tumefaciens* transformation protocols (Curtin et al., supra). Following cultivation of the T-DNA-containing *A. tumefaciens* strains with soybean half cotyledons (variety Bert) and subsequent selection for expression of bar, putatively transgenic plants were regenerated. The plants were transferred to soil, and after approximately 4 weeks of growth, a small leaf was harvested from each plant for DNA extraction and genotyping. Each DNA sample was first screened using PCR for the presence of bar. All bar-positive plants were then subjected to a T7E1 assay to identify plants with mutations at the FAD2-1A and FAD2-1B TAL effector endonuclease recognition site (Kim et al., *Genome Res.* 19:1279-1288, 2009). Briefly, a PCR product spanning the TAL effector endonuclease recognition site was generated, denatured, and allowed to reanneal. T7E1 endonuclease was added to the annealed products to cleave heteroduplexes generated when a wild type DNA fragment annealed with a fragment carrying a TAL effector endonuclease-induced mutation, and cleavage products were visualized by agarose gel electrophoresis. As shown in FIG. 4, four plants showed evidence of TAL effector endonuclease-induced mutations (Gm026-18, Gm026-23, Gm027-06 and Gm027-07). In addition, all four plants had mutations at both FAD2-1A and FAD2-1B, indicating that both genes were mutagenized simultaneously. The genotyping data for all plants regenerated is shown in Table 4B.

To determine if mutations introduced by TAL effector endonucleases in leaf tissue were transmitted to the next generation, seeds were collected from T0 plants Gm026-18, Gm026-23 and Gm027-06. In each T1 population, 20-60 individual plants were genotyped to confirm transmission of the mutations. Both FAD2-1A and FAD2-1B mutations segregated in the T1 progeny of GM026-18. In contrast, only FAD2-1A or FAD2-1B mutations were transmitted to the T1 progeny of GM-026-23 and GM027-6, respectively. The DNA sequences of the heritable mutations are shown in FIG. 5.

Example 5

Fatty Acid Profile Analysis on Seeds Produced from TAL Effector Endonuclease-induced Mutations in Soybean FAD2 Genes

Seed derived from soybean lines that are homozygous mutant in either FAD2-1A or FAD2-1B, or homozygous for

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both FAD2-1A and FAD2-1B, are analyzed for fatty acid composition. Briefly, individual soybean seeds derived from Gm026-23, Gm027-6, or Gm026-18 are pulverized individually. DNA is prepared from a portion of the ground tissue and is analyzed to confirm the genotype of each seed. Pulverized tissue from FAD2-1A homozygous (Gm26-23), FAD2-1B homozygous (Gm027-6), or FAD2-1A/FAD2-1B double homozygous (Gm026-18) knock out seeds is pooled. Fatty acid composition is then determined using fatty acid methyl esters (FAME) gas chromatography (Beuselinck et al., *Crop Sci.* 47:747-750, 2006), to assess whether seeds with various FAD2-1 mutations are altered in the proportion of linoleic acid and oleic acid relative to wild type seed.

TABLE 4A

List of regenerated plants and FAD2-1 genes analyzed in FIG. 4		
Lane	DNA	Gene
1	GM026-7a	FADII 1A
2	GM026-17	FADII 1A
3	GM026-18	FADII 1A
4	GM026-20	FADII 1A
5	GM026-23	FADII 1A
6	GM027-3	FADII 1A
7	GM027-6	FADII 1A
8	GM027-7	FADII 1A
9	GM027-10	FADII 1A
10	GM027-11	FADII 1A
11	GM008-6	FADII 1A
12	Bert WT	FADII 1A
13	GM026-7a	FADII 1A uncut
14	GM026-17	FADII 1A uncut
15	GM026-18	FADII 1A uncut
16	GM026-7a	FADII 1B
17	GM026-17	FADII 1B
18	GM026-18	FADII 1B
19	GM026-20	FADII 1B
20	GM026-23	FADII 1B
21	GM027-3	FADII 1B
22	GM027-6	FADII 1B
23	GM027-7	FADII 1B
24	GM027-10	FADII 1B
25	GM027-11	FADII 1B
26	GM008-6	FADII 1B
27	Bert WT	FADII 1B
28	GM026-7a	FADII 1B uncut
29	GM026-17	FADII 1B uncut
30	GM026-18	FADII 1B uncut

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TABLE 5

Representative FAD2-1A and FAD2-1B coding sequences*	
FAD2-1A	
5	ATGgtcatgatttcactctctctaatctctccattcattttgtagttg
	tcattatcttttagatttttctactacctgggtttaaattgagggattgt
10	agttctgttggtacatattacacattcagcaaaacaactgaaactcaa
	ctgaacttgtttatactttgacacagGGTCTAGCAAAGGAAACAACAA
	TGGGAGGTAGAGGTCGTGTGGCCAAAGTGAAGTTCAAGGGAAGAAGC
15	CTCTCTCAAGGGTTCAAACACAAAGCCACCATTCACTGTTGGCCAAC
	TCAAGAAAGCAATTCCACCACACTGCTTTCAGCGCTCCCTCCTCACTT
	CATTCTCCTATGTTGTTTATGACCTTTCATTTGCCTTCATTTTCTACA
20	TTGCCACCACCTACTTCCACCTCCTTCTCAACCCTTTTCCCTCATTG
	CATGGCCAATCTATTGGGTTCTCAAGGTTGCCTTCTCACTGGTGTGT
	GGGTGATTGCTCACGAGTGTGGTCACCATGCCTTCAGCAAGTACCAAT
25	GGGTTGATGATGTTGTGGGTTTGACCCCTTCACTCAACACTTTTAGTCC
	CTTATTTCTCATGGAAAATAAGCCATCGCCGCATCACTCCAACACAG
	GTTCCCTTGACCGTGATGAAGTGTGTTGTCCAAAACCAAATCCAAAG
30	TTGCATGGTTTTCCAAGTACTTAAACAACCCTCTAGGAAGGGCTGTTT
	CTCTTCTCGTCACACTCACAATAGGGTGGCCTATGTATTTAGCCTTCA
	ATGTCTCTGGTAGACCCTATGATAGTTTTGCAAGCCACTACCACCCTT
35	ATGCTCCATATATTCTAACCGTGAGAGGCTTCTGATCTATGTCTCTG
	ATGTTGCTTTGTTTTCTGTGACTTACTCTCTTACCCTGTTGCAACCC
	TGAAAGGGTTGGTTTTGGCTGCTATGTGTTTTATGGGGTGCCTTTGCTCA
40	TTGTGAACGGTTTTCTTGTGACTATCACATATTTGCAGCACACACT
	TTGCCTTGCCCTCATTACGATTCATCAGAATGGGACTGGCTGAAGGGAG
	CTTTGGCAACTATGGACAGAGATTATGGGATTCTGAACAAGGTGTTTC
45	ATCACATAACTGATACTCATGTGGCTCACCATCTCTTCTTACAATGC
	CACATTACCATGCAATGGAGGCAACCAATGCAATCAAGCCAATATTGG
	GTGAGTACTACCAATTTGATGACACACCATTTTACAAGGCACTGTGGA
50	GAGAAGCGAGAGAGTGCCTCTATGTGGAGCCAGATGAAGGAACATCCG
	AGAAGGGCGTGTATTGGTACAGGAACAAGTATTGA (SEQ ID NO: 38)

TABLE 4B

Mutant Screening in T0 Transgenic Soybean Plants										
	GM026-7a	GM026-17	GM026-18	GM026-20	GM026-23	GM027-3	GM027-6	GM027-7	GM027-10	GM027-11
FAD2-1A	Wt	Wt	Mutation	Wt	Mutation	Wt	Mutation	Mutation	Wt	Wt
FAD2-1B	Wt	Wt	Mutation	Wt	Mutation	Wt	Mutation	Mutation	Wt	Wt

*Wt signifies wild type genotype based on T7E1 assay

TABLE 5-continued

Representative FAD2-1A and FAD2-1B coding sequences*	
FAD2-1B	
ATGgtcatgatttcactetctetaatctgtcacttccetccattcatt	
ttgtacttetcatatTTTTcacttccctggttgaattgtagttctct	
tggtacatactagtagtagacattcagcaacaacaactgaactgaact	
tctttatactttgacacagGGTCTAGCAAAGGAAACAATAATGGGAGG	
TGGAGGCCGTGTGGCCAAAGTTGAAATTCAGCAGAAGAAGCCTCTCTC	
AAGGGTTCCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAA	
AGCCATTCCACCGCACTGCTTTTCAGCGTTCCCTCCTCACTTCATTGTC	
CTATGTTGTTTATGACCTTTCATTGGCTTTCATTTCTACATTGCCAC	
CACCTACTTCCACCTCCTCCCTCACCCCTTTCCCTCATTGCATGGCC	
AATCTATTGGGTTCTCCAAGGTTGCATTCTTACTGGCGTGTGGGTGAT	
TGCTCACGAGTGTGGTCACCATGCCTTCAGCAAGTACCCATGGGTTGA	
TGATGTTATGGGTTTGACCGTTCACCTCAGCACTTTTAGTCCCTTATTT	
CTCATGGAAAATAAGCCATCGCCGCCACCCTCCAACACGGGTTCCCT	
TGACCGTGATGAAGTGTGTTGTCCAAAACCAAATCCAAGTTGCATG	
GTACACCAAGTACCTGAACAACCTCTAGGAAGGCTGCTTCTCTTCT	
CATCACACTCACAATAGGGTGGCCTTTGTATTTAGCCTTCAATGTCTC	
TGGCAGACCCTATGATGGTTTTGCTAGCCACTACCACCCTTATGCTCC	

TABLE 5-continued

Representative FAD2-1A and FAD2-1B coding sequences*	
5	CATATATTCAAATCGTGAGAGGCTTTTGATCTATGTCTCTGATGTTGC
	TTTGTGTTTCTGTGACTTACTTGCTCTACCGTGTGCAACTATGAAAGG
	GTTGGTTTGGCTGCTATGTGTTTATGGGGTGCCATTGCTCATTGTGAA
10	CGGTTTTCTTGTGACCATCACATATCTGCAGCACACACACTATGCCTT
	GCCTCACTATGATTCATCAGAATGGGATTGGCTGAGGGGTGCTTTGGC
	AACTATGGACAGAGATTATGGAATTCTGAACAAGGTGTTTCACCACAT
15	AACTGATACTCATGTGGCTCACCATCTTTTCTCTACAATGCCACATTA
	CCATGCAACGGAGGCAACCAATGCAATGAAGCCAATATTGGGTGAGTA
	CTACCGATTTGATGACACACCATTTTACAAGGCACTGTGGAGAGAAGC
20	AAGAGAGTGCCTCTATGTGGAGCCAGATGAAGGAACATCCGAGAAGGG
	CGTGTATTGGTACAGGAACAAGTATTGA
	(SEQ ID NO: 39)

*intron sequences are in lower case

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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 cacgagtgtg gtcaccatgc cttcagcaag taccaatggg ttgatgatgt tgtgggtttg 180
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 cacgagtgtg gtcaccatgc cttcagcaag taccatggg ttgatgatgt tatgggtttg 180
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tgggttctcc aaggtt 76

<210> SEQ ID NO 4
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 <220> FEATURE:
 <223> OTHER INFORMATION: synthetic oligonucleotide

<400> SEQUENCE: 4

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<210> SEQ ID NO 5
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 <220> FEATURE:
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<400> SEQUENCE: 5

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tt 62

<210> SEQ ID NO 7
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 <213> ORGANISM: Glycine max

<400> SEQUENCE: 7

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 <220> FEATURE:
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 <212> TYPE: DNA
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<400> SEQUENCE: 20
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<400> SEQUENCE: 32
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 <213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 38

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<212> TYPE: DNA
<213> ORGANISM: Glycine max

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cagcaacaac aactgaactg aacttcttta tactttgaca cagggtctag caaaggaaac    180
aataatggga ggtggaggcc gtgtggccaa agtgaaatt cagcagaaga agcctctctc    240
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gcactgcttt cagegttccc tctcacttc attgtcctat gttgtttatg acctttcatt    360
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caaagttgca tggtagacca agtacctgaa caaccctcta ggaagggctg cttctcttct	720
catcacactc acaatagggt ggcccttgta tttagccttc aatgtctctg gcagacccta	780
tgatggtttt gctagccact accaccctta tgetccata tattcaaatac gtgagaggct	840
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cggttttctt gtgaccatca catatctgca gcacacacac tatgccttgc ctcactatga	1020
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ctatgtggag ccagatgaag gaacatccga gaagggcgtg tattggtaca ggaacaagta	1320
ttga	1324

What is claimed is:

1. A soybean plant, plant part, or plant cell comprising:
 - (a) a deletion in each FAD2-1A allele, wherein said deletion in each FAD2-1A allele was induced by transcription activator-like (TAL) effector endonucleases targeted to SEQ ID NOS: 32 and 33, and
 - (b) a deletion in each FAD2-1B allele, wherein said deletion in each FAD2-1B allele is 23 bp in size, and wherein said deletion in each FAD2-1B allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33,
 wherein oil produced from said plant, plant part, or plant cell has increased oleic acid content and decreased linoleic acid content as compared to oil produced from a corresponding wild type soybean plant, plant part, or plant cell.
2. The soybean plant, plant part, or plant cell of claim 1, wherein each said FAD2-1A and FAD2-1B allele has a deletion of an endogenous nucleic acid sequence and does not include any exogenous nucleic acid.
3. A soybean plant, plant part, or plant cell comprising:
 - (a) a deletion in each FAD2-1A allele, wherein said deletion in each FAD2-1A allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, and
 - (b) a deletion in each FAD2-1B allele, wherein said deletion in each FAD2-1B allele is 23 bp in size, and wherein said deletion in each FAD2-1B allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33,
 wherein oil produced from said plant, plant part, or plant cell has an oleic acid content of greater than 30%.
4. The soybean plant, plant part, or plant cell of claim 3, wherein said oleic acid content is greater than 40%.
5. A soybean plant, plant part, or plant cell comprising:
 - (a) a deletion in each FAD2-1A allele, wherein said deletion in each FAD2-1A allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, and
 - (b) a deletion in each FAD2-1B allele, wherein said deletion in each FAD2-1B allele is 23 bp in size, and wherein said deletion in each FAD2-1B allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33,
 wherein oil produced from said plant, plant part, or plant cell has a linoleic acid content of less than 10%.
6. The soybean plant, plant part, or plant cell of claim 5, wherein said linoleic acid content is less than 5%.
7. A method for producing soybean oil having increased oleic acid content and reduced linoleic acid content, comprising:
 - (a) providing a soybean plant or plant part comprising
 - (i) a deletion in each FAD2-1A allele, wherein said deletion in each FAD2-1A allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, and
 - (ii) a deletion in each FAD2-1B allele, wherein said deletion in each FAD2-1B allele is 23 bp in size, and wherein said deletion in each FAD2-1B allele was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33; and
 - (b) producing oil from said plant or plant part.
8. A method for making a soybean plant comprising a deletion in each FAD2-1A allele and a deletion in each FAD2-1B allele, wherein the deletion in each said FAD2-1B allele is 23 bp in size, said method comprising:
 - (a) contacting a population of soybean plant cells comprising functional FAD2-1A and FAD2-1B alleles with a TAL effector endonuclease targeted to SEQ ID NO: 32, and a TAL effector endonuclease targeted to SEQ ID NO: 33,
 - (b) selecting, from said population, a cell in which each FAD2-1A allele and each FAD2-1B allele comprises a deletion, wherein said deletion in each FAD2-1A allele was induced by said TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, wherein said deletion

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in each FAD2-1B allele was induced by said TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, and wherein said deletion in each said FAD2-1B allele is 23 bp in size, and

(c) regenerating said selected plant cell into a soybean plant. 5

9. The method of claim 8, wherein said soybean plant cells comprise cotyledon cells, and wherein said methods comprises transforming said cotyledon cells with one or more vectors encoding said TAL effector endonucleases. 10

10. The method of claim 8, comprising introducing into said plant cells one or more TAL effector endonuclease proteins.

11. The method of claim 8, further comprising culturing said plant cells to generate plant lines. 15

12. The method of claim 8, further comprising isolating genomic DNA comprising at least a portion of the FAD2-1A locus or at least a portion of the FAD2-1B locus from said plant cells.

13. A method for generating a soybean plant comprising a deletion in each FAD2-1A allele and a deletion in each FAD2-1B allele, wherein the deletion in each said FAD2-1B allele is 23 bp in size, said method comprising: 20

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(a) crossing a first soybean plant comprising a deletion in at least one FAD2-1A allele and a 23 bp deletion in at least one FAD2-1B allele with a second soybean plant comprising a deletion in at least one FAD2-1A allele and a 23 bp deletion in at least one FAD2-1B allele, to obtain progeny, wherein said deletion in at least one FAD2-1A allele in said first soybean plant was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, wherein said deletion in at least one FAD2-1B allele in said first soybean plant was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, wherein said deletion in at least one FAD2-1A allele in said second soybean plant was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33, and wherein said deletion in at least one FAD2-1B allele in said second soybean plant was induced by TAL effector endonucleases targeted to SEQ ID NOS: 32 and 33; and

(b) from said progeny a soybean plant that comprises a deletion in each FAD2-1A allele and each FAD2-1B allele.

* * * * *